EVIDENCE FOR REVERSIBLE PHOSPHORYLATION AS A POST-TRANSLATIONAL REGULATORY MECHANISM IN THE CHLOROPHYLL CATABOLIC PATHWAY

BY

PAMELA KRISTIN HALL

THESIS

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Adviser:

Professor Donald R. Ort

ABSTRACT

Senescence, the final developmental stage in plants, is a highly regulated degenerative process, which ultimately allows for the remobilization of nutrients within the plant from older to younger developing tissues and organs. Among the events characteristic of senescence in aerial plant tissues is the disassembly of the photosynthetic apparatus, accompanied by the release and degradation of chlorophyll molecules. As unbound chlorophyll and several of its catabolites react readily with light to form damaging reactive oxygen species, chlorophyll degradation functions as a critical detoxification process in senescing plants. Chlorophyll is rapidly catabolized into a set of linear, photodynamically safe compounds through a series of enzyme-mediated steps. Although these steps have all been identified, little is known about their regulation, particularly at the post-translational level. A better understanding of the post-translational mechanisms governing activity of one enzyme in particular, pheophorbide *a* oxygenase (PaO), is especially desirable as PaO catalyzes the oxidative opening of the porphyrin ring structure, considered to be the key regulatory point in chlorophyll degradation. Through site-directed mutagenesis of the *Arabidopsis thaliana* gene encoding PaO, the *in vivo* effects of the phosphorylation state at a defined site on this enzyme were explored. The mutant plant line made to mimic the dephosphorylated state of PaO at the specified position exhibited slightly accelerated rates of chlorophyll degradation and was fully capable of catabolizing pheophorbide *a*, the substrate of PaO. The plant line mimicking the phosphorylated state at this same position behaved very differently; chlorophyll degradation was not noticeably affected, however, the ability of PaO to catabolize pheophorbide *a* was drastically reduced, as indicated by quantification of this catabolite in

senescing leaf tissue. Thusly, it could be concluded from the analyses of chlorophyll content and catabolite pools throughout senescence in these mutant Arabidopsis lines, that phosphorylation at a defined position on this enzyme greatly attenuates PaO activity, indicating a role for reversible phosphorylation in regulating PaO.

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CHAPTER 1

INTRODUCTION

Senescence represents the final stage of plant development and is characterized by several highly regulated degenerative processes. A key component of senescence, the degradation of cellular proteins, ultimately allows for the release and remobilization of important nutrients, such as nitrogen and phosphorus (Hörtensteiner and Feller, 2002), from a plant's senescing leaves to its younger developing organs, namely new leaves, seeds, and fruits (Gan and Amasino, 1997). Events involved in senescence include the structural disorganization of chloroplasts, disassembly of the photosynthetic apparatus, and subsequent degradation of chlorophyll molecules (Noodén et al., 1997).

Chlorophyll degradation is the most visually apparent indication of senescence, and it is estimated that one billion tons of chlorophyll are degraded annually (Hendry et al., 1987). The breakdown of chlorophyll molecules has an integral role during senescence, as it not only contributes to nutrient recycling within the plant, but functions as a critical detoxification process in the senescing tissues as well. Chlorophyll molecules released from the thylakoid membrane-bound light harvesting complexes during senescence are highly photoactive and must be catabolized rapidly to prevent the formation of damaging, cytotoxic reactive oxygen species (ROS) in the presence of light (Apel and Hirt, 2004; Hörtensteiner, 2004). Furthermore, several intermediate compounds formed during chlorophyll degradation are also potential strong phototoxins. As such, the existence of a highly regulated, rapid, and efficient catabolic process to dispose of these cytotoxic compounds is a necessity.

In crop species in particular, the significance of chlorophyll degradation during senescence extends beyond that of its role in nutrient recycling and detoxification, as this process can potentially have considerable agronomic implications as well. So-called stay-green mutants, in which chlorophyll degradation is either delayed or interrupted at some level, have been identified in many species of higher plants (*e.g.*, Bachmann et al., 1994; Vincentini et al., 1995; Cha et al., 2002). Depending on the type and location of the mutation responsible, the stay-green phenotype can have either advantageous or detrimental effects on agronomic traits, ultimately influencing crop yield and seed quality. Namely, in stay-green mutants classified as 'functional,' photosynthesis is prolonged, potentially leading to increases in crop productivity; by contrast, mutants classified as 'cosmetic' have lost their photosynthetic capacity despite retaining photosynthetic pigments, and thus lack the potential to contribute to yield increases (Thomas and Howarth, 2000). Furthermore, some cosmetic stay-green mutants are the result of defects in the chlorophyll degradation pathway that lead to the accumulation of phototoxic catabolites in senescing tissues, oftentimes having a substantially negative impact on productivity.

The potential for certain lesions in the chlorophyll catabolic process to have deleterious effects on agronomic traits is well-exemplified in the canola industry, by the so-called "green seed problem." That is, when canola plants experience a freezing episode during development, chlorophyll is often retained at undesirably high levels in the seeds throughout senescence (Johnson-Flanagan and Thiagarajah, 1990). Such an event, which commonly occurs in regions of northern latitudes where canola is widely grown, can result in major economic losses for the commercial canola industry, as residual phototoxic

chlorophyll must be removed from seed oil through a tedious and costly process (Levadoux et al., 1987). The potential for chlorophyll degradation to impact crop productivity, both positively and negatively, underscores the necessity for developing a concise understanding of the mechanisms controlling this catabolic pathway.

Until relatively recently, research on chlorophyll metabolism had focused almost exclusively on biosynthesis, leaving chlorophyll catabolism to be considered a "biological enigma" (Hendry et al., 1987). Studies conducted primarily in the past two decades have elucidated much of the chlorophyll degradation pathway, revealing that it is a tightly regulated catabolic process occurring through a series of predominantly enzyme-mediated steps (Hörtensteiner, 2006). The final products of chlorophyll degradation comprise a group of linear, colorless tetrapyrroles, classified as non-fluorescent chlorophyll catabolites, or NCCs (Hörtensteiner, 1999). With the exception of one NCC found in Arabidopsis (Müller et al., 2006), these photodynamically safe end products are derived exclusively from chlorophyll *a*, as chlorophyll *b* derivatives act as competitive inhibitors at various steps along the described degradation pathway (Hörtensteiner, 2006). Thus chlorophyll *b* is degraded by conversion to chlorophyll *a* (Folley and Engel, 1999).

Upon initiation of senescence, the chlorophyll-binding proteins of the photosynthetic apparatus disassemble, leading to the eventual release of chlorophyll molecules (Hilditch et al., 1989). Analyses of mutant plants lacking expression of the gene denoted *STAY-GREEN (SGR)* have determined that the protein SGR likely plays a critical role in mediating the removal of chlorophyll from apoproteins at light harvesting complex II (LHCII), in particular (Park et al., 2007; Hörtensteiner, 2009). Following this release of chlorophyll molecules from the chloroplast apoproteins, chlorophyll *b* must be converted

to chlorophyll *a* (Scheumann et al., 1999). This conversion is mediated by the enzymes NYC1 (non-yellow coloring1) and NOL (non-yellow coloring1-like), which comprise two subunits of chlorophyll *b* reductase and thus act in tandem to catalyze the first portion of the conversion of chlorophyll *b* to chlorophyll *a* at the thylakoid membrane (Kusaba et al., 2007; Sato et al., 2009).

It was initially determined that the next step in the pathway was the removal of the phytol tail of chlorophyll *a* by the enzyme chlorophyllase (Jacob-Wilk et al., 1999; Tsuchiya et al., 1999); however, ambiguous results from recent studies, such as localization of several chlorophyllase enzymes to areas outside of the chloroplast (Takamiya et al., 2000), have since eliminated this from consideration as a necessary enzyme in the chlorophyll degradation pathway (Schenk et al., 2007). It has recently been demonstrated that removal of the central Mg^{2+} atom must precede the removal of the phytol tail. Mg^{2+} is removed from the porphyrin ring of chlorophyll by an unknown metal chelating substance. A putative gene for this chelator, which coexpresses with genes responsible for downstream steps in the chl degradation pathway, has recently been identified in Arabidopsis (Lundquist et al., 2012). Subsequently, pheophytinase (PPH), rather than chlorophyllase, is responsible for the removal of the phytol tail from the Mg^{2+}-free pheophytin *a* molecule (Schelbert et al., 2009).

Separation of the phytol tail from the porphyrin macrocycle results in the formation of pheophorbide *a.* This final tetrapyrrolic ring structure in the chlorophyll degradation pathway is then opened by the introduction of an oxygen atom at the α-meso position of the compound (Rodoni et al., 1997). As this reaction results in the loss of green color, it is thought to be a very important step in the chlorophyll catabolic pathway. Pheophorbide *a*

oxygenase (PaO), a nonheme monooxygenase, is the enzyme responsible for the oxidative opening of the porphyrin ring (Hörtensteiner et al., 1998), and it has been shown in plants across various species that interference with the function of this enzyme results in two distinct phenotypes (Pružinská et al., 2003, 2005). Under normal light conditions, interruption of PaO expression results in a lesion-mimic phenotype (Gray et al., 2002; Tanaka et al., 2003), characterized by the formation of cell death lesions on leaf surfaces, as seen in plants containing the *AtPaO* mutant allele, *accelerated cell death1* (*acd1*) (Pružinská et al., 2003). This phenotype occurs in an age-dependent manner, with the oldest rosette leaves developing cell death lesions the earliest. These cell death lesions are caused by the accumulation of pheophorbide *a*, the phototoxic substrate of PaO, in leaves of mutant plants lacking appropriate PaO expression. Under dark conditions, such as during artificially-induced senescence, these same mutants exhibit a stay-green phenotype (Pružinská et al., 2005), the result of the retention of either undegraded chlorophyll or green chlorophyll catabolites, or a combination of these compounds, in senescing leaf tissue. PaO mutants are classified as type C 'cosmetic' stay-green mutants, because although chlorophyll catabolism is impaired, other senescence processes occur as usual and leaves are unable to perform photosynthesis (Thomas and Howarth, 2000).

The product of the oxygenolytic opening of the porphyrin ring by PaO is a linearized tetrapyrrolic compound, called red chlorophyll catabolite (RCC) (Rodoni et al., 1997). This compound is particularly unstable and is rapidly reduced, in the presence of reduced ferredoxin from the oxidative pentose phosphate pathway, by the enzyme RCC reductase (RCCR) (Wüthrich et al., 2000). Constitutive activity of the RCCR protein throughout leaf development has been demonstrated in Arabidopsis (Mach et al., 2001). The reduction

step results in the formation of a group of non-colored, but blue-fluorescing, primary chlorophyll catabolites (pFCCs), stereoisomers whose configuration at the C1 chiral center depends on the stereospecificity of RCCR (Pružinská et al., 2007). The gene encoding the enzyme RCCR, *Accelerated cell death2 (ACD2)*, has been cloned and characterized in Arabidopsis, as well as in several other species (Mach et al., 2001). *acd2-2* functional knockout mutants of *Arabidopsis thaliana* are known to display a cell death mimic phenotype (Greenberg et al., 1994; Mach et al., 2001), similar to that observed in *acd1* plants, however, the cell death lesions in *acd2* leaves are caused by accumulation of RCC, the phototoxic substrate of RCC reductase, rather than by pheophorbide *a* (Pružinská et al., 2007).

The set of primary fluorescent chlorophyll catabolites formed by the reduction of RCC, though no longer photoactive, are not the end products of chlorophyll degradation. pFCCs are exported from the senescing plastids to the cytosol, where they receive modifications at one or several side positions (Matile et al., 1999). The modified fluorescent catabolites are then transported into the cell vacuole by an unidentified primary active transport system (Hinder et al., 1996), possibly involving a group of ATP binding cassette (ABC)-type transporters (Tommasini et al., 1998; Jonker et al., 2002). Once inside the cell vacuole, the acidic environment catalyzes the non-enzymatic isomerization of pFCCs to yield non-fluorescent, colorless catabolites (NCCs) (Oberhuber et al., 2003). The first NCC determined to be the final product of chlorophyll degradation was identified and isolated in barley (*Hordeum vulgare*) (Kraütler et al., 1991), and many more NCCs have since been identified across a range of higher plants (Matile et al., 1999). These

photodynamically safe end-products of chlorophyll catabolism are permanently stored in the vacuoles of the senescing leaf cells (Matile et al., 1988).

In spite of the progress made over the last two decades in defining the steps of chlorophyll degradation, an understanding of the regulation of each step, particularly at the post-translational level, remains largely limited. While gene expression and transcriptional regulation of the catabolic steps have received considerable attention (Gepstein et al., 2003; Guo et al., 2004; Buchanan-Wollaston et al, 2005), to date, regulation of the enzymes encoded by these genes is a relatively unexplored area. A better understanding of the post-translational level of regulation of the chlorophyll degradation pathway is crucial, as the possibility exists that it is at this level that the effects of certain biotic and abiotic stress factors on chlorophyll catabolism might be the most pronounced.

As pheophorbide *a* oxygenase mediates what is thought to be the pivotal regulatory step in chlorophyll degradation, it seems a reasonable candidate for investigation into post-translational regulatory mechanisms governing the chlorophyll catabolic process. PaO is a 52 kDa protein classified as a non-heme monooxygenase, containing the characteristic Rieske-type domain and mononuclear iron-binding domain (Gray et al., 2004). Additionally, the PaO protein contains a chloroplast transit peptide, two transmembrane domains, two putative calcium-dependent protein kinase (CDPK) target sites, as well as a thioredoxin target site (Fig. 1). The protein was originally believed to localize to the chloroplast inner envelope membrane (Matile and Schellenberg, 1996), a notion that was later confirmed by proteomic analyses in Arabidopsis (Joyard et al., 2009). A more recent study, however, used *in vitro* and *in vivo* pull-down assays of PaO linked to the green fluorescent protein (GFP) to demonstrate that PaO may actually localize to the chloroplast

thylakoid membrane, where it would presumably interact and form a complex with several other catabolic enzymes to rapidly channel chlorophyll and its phototoxic intermediates through the degradation pathway (Sakuraba et al., 2012). Expression of PaO at both the gene and protein levels has been demonstrated to occur in a senescence-dependent pattern, and, under normal, non-stressed conditions, activity of the enzyme appears to follow this expression pattern (Pružinská et al., 2003, 2005).

A study conducted by Chung et al. in 2006, however, suggested that a disconnect exists between PaO expression and activity, as demonstrated in canola (*Brassica napus*) seeds exposed to freezing temperatures. The object of their study was to determine the probable step in chlorophyll degradation responsible for the previously described green seed problem in canola. Chung et al. found that, under treatment conditions, chlorophyll degradation was interrupted, and an accumulation of pheophorbide *a*, PaO's substrate, was observed. Expression of PaO, at both the transcript and protein levels, was not affected by the cold stress; PaO activity, on the other hand, appeared to be significantly reduced (>20%) in seeds receiving the cold treatment compared with those in a control group. Their finding suggests that PaO is perhaps most strongly regulated at the post-translational level, and that further investigation into possible mechanisms of post-translational regulation may be key to better understanding this important step in the chlorophyll degradation process. Their study went on to show that reduced enzyme activity correlated with an increase in the phosphorylated fraction of PaO, providing preliminary evidence in support of a role for a phosphorylation mechanism in the regulation of PaO activity, particularly in temperature-stressed plants (Chung et al., 2006).

Further investigation into this role of a phosphorylation mechanism in activating/deactivating PaO is therefore in order, as an understanding of this mechanism could ultimately provide insight into the basis of the green seed problem, and, perhaps of more immediate relevance, into the regulation of chlorophyll catabolism as a whole. In plants, there are five classified major protein kinase families known to mediate the transfer of a phosphate group from ATP to an amino acid side chain on a protein (Stone et al., 1995). The hyperphosphorylated state of PaO that Chung et al. observed in freeze–treated canola plants can most likely be attributed to the kinase group referred to as the calcium-dependent protein kinases (CDPKs). CDPKs, which phosphorylate at threonine/serine residues on proteins, require the binding of calcium to an EF-hand domain in order to activate the catalytic domain of the kinase (Harmon et al., 2001). Cool temperatures are known to trigger transient Ca^{2+} increases in plant cells (Gilroy and Trewavas, 1994), so CDPKs would predictably be found in a highly active state in plants receiving such treatment conditions (Martin and Busconi, 2001). As such, it seems reasonable to focus, in particular, on the effects of phosphorylation at candidate CDPK recognition sites on the PaO enzyme.

The objective of this study was to determine how the phosphorylation status at a defined site on pheophorbide *a* oxygenase affects both metabolic activity of this enzyme and, subsequently, the chlorophyll degradation process as a whole, during leaf senescence in *Arabidopsis thaliana*. Site-directed mutants mimicking the phosphorylated and nonphosphorylated state of PaO at Thr-404 displayed altered degrees of chlorophyll degradation and pheophorbide *a* accumulation *in vivo*, when compared to wild-type Arabidopsis plants. The results of this study provide convincing evidence to suggest that a

phosphorylation mechanism at Thr-404 on the PaO enzyme does indeed play a role in the regulation of the chlorophyll catabolic pathway. Further work is necessary to evaluate the importance of this post-translational modification in governing enzyme activity, relative to, or in conjunction with, other potential post-translational modifications.

CHAPTER 2

METHODOLOGY

2.1 Plant Material and Growth Conditions

Arabidopsis thaliana wild-type (Columbia (Col-0) ecotype) and transgenic seeds were sterilized and sown on flats containing Sunshine Mix LC1 soil (SunGro Horticulture). After 48 hours of stratification at 4° C, flats were moved to a growth chamber with relative humidity of 60% and light intensity of 100 μmol photons m^{-2} s^{-1}. Chambers were set to either a short-day (8-hour light/16-hour dark) or a long-day (16-hour light/8-hour dark) photoperiod, and a thermoperiod of 22°C-day/18°C-night. Following germination, plants were grown under the conditions described for eight weeks (short-day grown plants) or three to four weeks (long-day grown plants) prior to inducing artificial senescence.

2.2 Site-Directed Mutagenesis

Amino acid substitutions were introduced at the region of sequence encoding the threonine-404 residue on the *Pao* gene (At3g44880.1) using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and *PfuUltra* High-Fidelity DNA polymerase (Agilent Technologies, Santa Clara, CA, USA). This amino acid residue was selected for mutagenesis as it lies within a putative CDPK recognition site that was identified previously (Chung et al., 2006). Primers were designed to contain the desired mutations targeted to this putative CDPK phosphorylatable site, on the enzyme: PaO-P forward (5'-CGACGTGAACAAACAGTACGAGAAGCTCACATTCACTCCAAC–3'); PaO-P reverse (5'–GTTGGAGTGAATGTGAGCTTCTCGTACTGTTTGTTCACGTCG–3'); PaO-NP forward (5'–CGACGTGAACAAACAGTACGCTAAGCTCACATTCACTCCAAC–3'); PaO-NP reverse

(5'–GTTGGAGTGAATGTGAGCTTAGCGTACTGTTTGTTCACGTCG–3'). Mutagenesis PCR conditions were as follows: 95°C for 30 s, then 16 cycles of 95°C for 30 s, 65°C for 1 min, 68°C for 6 min. Presence of the mutations was confirmed by sequencing the *AtPaO* gene in both the 5' and 3' directions.

2.3 Gateway Cloning

Site directed mutants were PCR amplified using Phusion High-Fidelity DNA polymerase and the cycling parameters described. After an initial denaturation at 98°C for 30 s, samples underwent 25 cycles of the following: 98°C for 10 s; 65°C for 20 s; 72°C for 15 s. This was followed by a final extension at 72°C for 10 min. Primers used to create the blunt-ended PCR product were: pENTR-PaO forward (5'-CACCATGTCAGTAGTTTTACTCTC-3') and pENTR-PaO reverse (5'-CTCGATTTCAGAATGTACATAATCTCT-3'). The PCR product was TOPO cloned into the Gateway directional TOPO entry vector pENTR/D-TOPO (Invitrogen, Grand Island, NY, USA) and introduced into One Shot TOP10 chemically competent *Escherichia coli* cells. Kanamycin-resistant colonies were PCR-screened for presence of the plasmid. An LR recombination reaction using Gateway LR Clonase II was performed to transfer the gene from the pENTR vector to the selected Gateway-compatible destination vector, pEarleyGate100 (Earley et al., 2006), for expression in Arabidopsis.

2.4 Plant Transformation and Selection

The plant binary expression vector containing *PaO* in its mutated (NP and P) and unaltered forms was introduced into the *Agrobacterium tumefaciens* gentamycin-resistant strain, GV3101. The plasmids were introduced into the *pao1* T-DNA confirmed homozygous

knockdown line of *Arabidopsis thaliana* (SALK_111333c, TAIR) via *Agrobacterium tumefaciens* using the previously described floral dip method (Clough and Bent, 1998). Plants transformed with the empty pEarleyGate100 vector were employed as a negative control group, while those transformed with the wild-type *PaO* coupled to the CaMV 35s promoter were used as the positive, overexpresser control group. Transformants carrying the pEarleyGate100 vector, which contains a gene for BASTA herbicide resistance, were selected on soil by periodic application (every two days for ~two weeks) of the herbicide beginning 7 days after germination. After 3 generations of herbicide selection, segregation was no longer observed in the transgenic lines. Genomic DNA from rosette leaf tissue of individual plants was screened via PCR to verify presence of the transgene.

2.5 Artificial Senescence Induction

Senescence was induced in rosette leaves that were at identical developmental stages by detaching the leaf and incubating it in complete darkness for 6 to 7 days. Petioles were submerged in distilled water to maintain a constant transpiration stream. Alternatively, senescence was induced in attached leaves by covering the individual rosette leaves of plants growing in chambers with aluminum foil sleeves to prevent light from reaching the leaf (Weaver and Amasino, 2001). Foil sleeves were removed after either 3 days or 7 days, and leaves were re-exposed to chamber light conditions for a period of 1 day.

2.6 Chlorophyll Extraction and Quantification

Chlorophyll was extracted from individual leaf punches (1 cm diameter) and concentrations were calculated on a per leaf area ($\mu mol\ m^{-2}$) basis. Chlorophyll was

extracted from leaf tissue using chilled 100% methanol, and concentrations were determined spectrophotometrically with a microplate reader. Chlorophyll *a, b*, and total chlorophyll content were all calculated according to the set of absorption coefficients proposed by Lichtenthaler et al. (1990). Chlorophyll measurements were taken daily throughout the course of artificial senescence, giving 7 total time points.

2.7 Extraction of Green Chlorophyll Catabolites

Green polar chlorophyll catabolites were extracted from leaf tissue as described by Pružinská et al. (2005). One leaf disc (1 cm diameter) was ground in liquid nitrogen. Tissue was dissolved in 100 uL of 0.5 M Tris-HCl (pH 8.0), and 400 uL acetone was added to the solution. Samples were centrifuged (16,000g for 2 minutes) and supernatant was removed and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.8 Analysis of Chlorophyll Catabolites

Pheophorbide *a* in the supernatant of the extractions described above was identified and quantified by high performance liquid chromatography-mass spectrometry/mass-spectrometry (HPLC-MS/MS) performed on an Applied Biosystems 5500 QTrap with Agilent 1200 LC at the Metabolomics division of the Roy J. Carver Biotechnology Center (University of Illinois at Urbana-Champaign, Urbana, IL). Pheophorbide *a* concentrations in senescing leaf tissue were considered to be an indirect measure of PaO activity.

2.9 Statistical Analysis

Chlorophyll and pheophorbide *a* data were analyzed by a mixed model ANOVA using PROC MIXED (SAS Institute) to test for significant differences between plant genotypes. Genotype was treated as a fixed factor, while replicate was considered a random factor.

CHAPTER 3

RESULTS

3.1 Creation and confirmation of mutant Arabidopsis lines

Two putative CDPK phosphorylatable sites have been identified on pheophorbide *a* oxygenase, which appear to be conserved in both Arabidopsis and canola (Chung et al., 2006). The first of these two sites, containing the phosphorylatable residue serine-18, lies within a putative chloroplast transit peptide at the N-terminal region of the protein (Fig. 1). Following synthesis, the transit peptide sequence containing the first CDPK site is cleaved during transport into the chloroplast; this greatly reduces the likelihood that a phosphorylation mechanism at this site would play a role in enzyme regulation. The second CDPK site, threonine-404 (Thr-404), however, is situated near the C-terminal region of the protein (Fig. 1), and could easily be phosphorylated and dephosphorylated once inside the chloroplast. Thr-404 of AtPaO was thus selected as the target residue for studying the effects of phosphorylation state on enzyme activity and the chlorophyll degradation process during senescence.

In order to directly study the effects of phosphorylation at this site, the codon representing the Thr-404 amino acid residue was manipulated via site-directed mutagenesis on the *PaO* gene of *Arabidopsis thaliana*. Threonine was substituted with either alanine – a non-phosophorylatable residue – to mimic the dephosphorylated state, or glutamic acid – a negatively charged amino acid – to mimic the phosphorylated state. The mutated forms of the *AtPaO* gene, under control of the CaMV 35s constitutive promoter, were introduced back into *Arabidopsis pao1* T-DNA knockdown mutants (ecotype Col-0). Transgenic lines were designated PaO-NP (non-phosphorylated at Thr-404) and PaO-P

(phosphorylated at Thr-404). Two control lines were created by transforming plants with the empty cloning vector (designated EV), as well as the native form of *AtPaO*, overexpressed by coupling it to the CaMV 35s promoter (designated OX).

3.2 Rate and extent of chlorophyll degradation were altered by the phospho-site substitutions on PaO

Arabidopsis rosette leaves at identical developmental stages were sampled either from plants grown under long-day light conditions, at three to four weeks after germination, or from plants grown under short-day conditions, at eight weeks after germination. Senescence was induced artificially by incubating the leaves in complete darkness for a period of six days. Leaf punches were taken daily throughout the time course of senescence (T_0-T_6), and stored at -80° C until processed. All samples were processed simultaneously, and total chlorophyll content on a per leaf area basis was determined spectrophotometrically, using the established absorption coefficients (Lichtenthaler et al., 1990).

Chlorophyll content was similar among all Arabidopsis lines sampled prior to the induction of senescence (Fig. 2), indicating that chlorophyll biosynthesis was not affected in the mutant lines. Because chlorophyll content was initially the same in all samples, any observed differences in leaf chlorophyll between lines during senescence could be reasonably attributed to variations in the overall capacity of the chlorophyll degradation pathway, rather than to baseline inconsistencies in chlorophyll content.

After only one day in darkness, differences in chlorophyll content among the lines could be observed (Fig. 2). Average total chlorophyll levels in the PaO-NP line were

considerably lower than those seen in all other lines after one day, and as senescence progressed, chlorophyll content in this mutant line continuously decreased to levels lower than most, if not all, other lines at each timepoint measured. The final chlorophyll concentration in PaO-NP leaves, sampled after six days in darkness, was significantly lower than that observed in all other plant lines ($P < 0.05$). These results suggest that chlorophyll is being catabolized slightly more rapidly in the PaO-NP mutants compared to the other plant lines.

The effects of the PaO-P mutation on the chlorophyll degradation pathway were not readily identifiable from the chlorophyll content measurements. Chlorophyll levels in this line were not consistently higher, when compared to the other lines, during senescence; however, late in the senescence regime, at days five and six, chlorophyll levels were higher than those seen in PaO-NP plants, but no statistically significant difference from the wild-type Col-0 plants was seen (Fig. 2). As would be expected, chlorophyll degradation was initially delayed in the EV parental control line, demonstrating that a PaO deficiency may, in fact, cause a delay in chlorophyll degradation.

The measured differences in rate and extent of chlorophyll degradation among plant lines suggest that the phospho-site substitutions at Thr-404 of PaO had an effect on the chlorophyll catabolic process. These data imply that PaO is slightly more active when Thr-404 is in a dephosphorylated state, as was evidenced by the observed acceleration in chlorophyll degradation in the PaO-NP line. Whether PaO activity is attenuated by phosphorylation at the same site, as would be predicted, was not discernable from the chlorophyll data alone.

3.3 Pheophorbide a accumulated in PaO-P, but not PaO-NP, leaves during senescence

Leaf punches from the artificial senescence assays described above were also taken for analyzing concentrations of the chlorophyll catabolite pheophorbide *a*. Pheophorbide *a* is the substrate of PaO, and is sequentially the last cyclic catabolite in the chlorophyll degradation pathway. Because PaO directly catalyzes the breakdown of this compound, measures of pheophorbide *a* concentration in leaf tissue throughout senescence can be used as an indirect means of quantifying PaO activity. Accumulation of pheophorbide *a* in senescing leaves compared to the control would reliably indicate an attenuation of PaO activity.

Catabolites were extracted from leaf punches and quantified by high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). Prior to senesence induction, at 0 days in darkness, levels of pheophorbide *a* were similar in the Arabidopsis lines assayed (Fig. 3), indicating that natural low-level chlorophyll turnover of recently expanded leaves was not affected in the mutant lines. A statistical difference between the OX and PaO-P lines was noted ($P < 0.05$) at day 0, however, because each of these lines were similar to the other three measured, and because such a dramatic difference between these two lines was subsequently observed, this initial difference could be disregarded. After 3 days in darkness, as expected, pheophorbide *a* concentrations had dropped in the wild-type and OX control lines, suggesting induction of PaO activity levels as senescence progressed. A similar decrease in pheophorbide *a* concentration between days 0 and 3 was also measured in the PaO-NP leaves, providing strong evidence that the phospho-site substitution mimicking dephosphorylation at Thr-404 does not attenuate PaO activity. Pheophorbide *a* levels in the EV parental control line did not change after 3 days in

darkness, which likely is the result of a known delay in induction of PaO activity by dark incubation (Chung et al., 2006).

After 5 and 7 days in darkness, pheophorbide *a* continued to be effectively catabolized in the PaO-NP, wild-type, and OX lines, as evidenced by a steady reduction in concentration (Fig. 3). After 5 days in darkness, pheophorbide *a* in the EV line had also decreased to levels similar to Col-O, OX, and PaO-NP. At 7 days in darkness, when the course of artificially-induced senescence was complete, pheophorbide *a* concentrations in all lines, with the exception of PaO-P, had aligned at around 2 μmol m^{-2}.

A markedly distinct result was observed in the PaO-P phosphorylation mimic line (Fig. 3). The divergence of pheophorbide *a* levels in the PaO-P line from those of the other plant lines studied was first detected, and most dramatic, midway through the dark incubation period. Beginning with the first of three timepoints sampled following senescence induction (3 days in darkness), pheophorbide *a* averages were significantly higher ($P < 0.0001$) in PaO-P leaves compared with each of the other lines. Between 0 and 3 days in darkness, pheophorbide *a* concentrations in these leaves had increased roughly 14-fold, clearly indicating that PaO activity in the PaO-P mutant line was reduced, as the enzyme catabolizing pheophorbide *a* was not able to keep pace with the rate at which its substrate was being formed. After the initial increase observed on day 3, pheophorbide *a* content in the PaO-P line began to drop, as seen at day 5, though levels were not significantly lower than those measured at day 3. This trend, marked by very slight decreases in pheophorbide *a* concentration following the intense increase at day 3, was observable during the remainder of senescence in PaO-P leaves. Despite the slight decreases detected between days 3 and 7, pheophorbide *a* was retained at very high levels

in fully senesced leaves. The final concentration of pheophorbide *a* measured in PaO-P leaves was nearly 10-fold higher than the initial concentration.

The pronounced differences in pheophorbide *a* levels between PaO-P and all other lines demonstrate that PaO is not fully functional during senescence in this particular mutant line, which strongly implies that phosphorylation at Thr-404 attenuates activity of this enzyme to an appreciable extent.

3.4 Retention of green-colored catabolites was visually detectable in PaO phosphorylation mutants

To determine whether mutant lines showed distinct visible differences during dark-induced senescence, attached Arabidopsis leaves were covered with aluminum foil sleeves to block light (Weaver and Amasino, 2001). Foil sleeves were left on for a period of three (mid-senescence), or seven (end of senescence) days. Following removal of foil sleeves, leaves were re-exposed to the same light conditions under which the plants were grown for one day, and leaf appearances were compared. Col-0 and PaO-NP leaves had begun to yellow after 3 days in darkness, although PaO-NP leaves appeared to be degreening considerably more rapidly than the wild-type leaves (Fig. 4, A and B). PaO-P leaves, by contrast, did not show signs of yellowing, but rather were lighter green in color and had a distinct dry and brittle appearance (Fig. 4C). These differences in appearance noted at 3 days in darkness between the PaO-P mutant and the other lines were even more exaggerated in leaves incubated for 7 days. PaO-NP and the wild-type control lines had lost nearly all of their green color by the end of the artificial senescence regime (Fig. 4, D and E),

whereas the PaO-P leaves remained a light green color and were more brittle than leaves only covered for 3 days (Fig. 4F).

Results from the analyses of total chlorophyll content and pheophorbide *a* content offer insight into the distinct appearance of PaO-P senescing leaves. Chlorophyll data showed that after 3 and 7 days in darkness, total chlorophyll levels in PaO-P leaves had dropped, suggesting that chlorophyll degradation was at least initiated in PaO-P leaves (Fig. 2). Despite this observation, the light green color retained in the dark-incubated leaves indicates a presence of undegraded chlorophyll in the leaf tissue. Alternatively, the presence of a green-colored catabolite could also be contributing to the green appearance of senescing PaO-P leaves. Because the LC-MS/MS data show the green chlorophyll catabolite pheophorbide *a* accumulating at such exceptionally high levels in PaO-P plants (Fig. 3), it seems feasible that this compound could affect the visual appearance of these leaves. Accordingly, the brittle appearance of the leaves, which suggests that some degree of membrane ion leakage – an early sign of cell death – had occurred, could be attributed to the phototoxicity of pheophorbide *a* (Hirashima et al., 2009), which had accumulated in the leaf tissue.

3.5 Mutant lines displayed light-dependent cell death in naturally senescing whole plants

Mutant, wild-type, and control plants growing in chambers under the described light conditions had indistinguishable phenotypes throughout germination and all subsequent developmental stages, prior to senescence (Fig. 5, A-C). The plants were visually identical and, as previously indicated, leaves of a similar age from each line sampled had statistically

comparable concentrations of both total chlorophyll and pheophorbide *a* before senescence had commenced.

When full plants were allowed to naturally enter the senescence stage in pots in growth chambers, control and mutant lines displayed considerable differences in appearance (Fig. 5 D-F). Rosette leaves of the Col-0 wild-type line showed some yellowing in a leaf age-dependent manner, beginning with the oldest leaves, as the plants began to flower (Fig. 5D). Unexpectedly, both PaO-P and PaO-NP mutant lines exhibited cell death lesions on senescing rosette leaves, which had also developed sequentially in a pattern dependent on individual leaf age (Fig. 5, E and F). By the time seed set had occurred, rosette leaves on PaO-P and PaO-NP plants were vastly, if not wholly, consumed by these regions of cell death.

Formation of cell death lesions in the mutant lines can almost certainly be attributed to the presence of photoactive compounds in the senescing leaves. Because the chlorophyll degradation pathway was manipulated by altering enzyme activity in these mutant lines, chlorophyll-derived catabolites presumably accumulated in the leaf tissue, where they reacted with light to form damaging reactive oxygen species, leading to cell death. In the case of the phosphorylation mimic mutant line (PaO-P), pheophorbide *a*, the substrate of PaO, accumulated at very high levels in senescing leaves, as was proven by LC/MS-MS quantification (Fig. 3). As PaO activity is impaired in this line, pheophorbide *a* was not catabolized during chlorophyll degradation and thus remained in the leaf tissue of naturally senescing plants, eventually resulting in cell death in the presence of light.

Analysis of pheophorbide *a* levels in the PaO-NP line, however, indicated that PaO does effectively catabolize this phototoxic compound (Fig. 3), eliminating it from

consideration as a possible cause of cell death in these mutant plants. If pheophorbide *a* was not responsible for the cell death phenotype observed in the PaO-NP line, the probable cause is another phototoxic catabolite in the chlorophyll degradation pathway. Catabolites upstream of pheophorbide *a* are also phototoxic, however, because pheophorbide *a* is initially present in PaO-NP leaves at levels comparable to those of wild-type leaves, and is subsequently catabolized at a rate similar to that seen in wild-type, it is very unlikely that any catabolites upstream of pheophorbide *a* would be accumulating in the leaf tissue of PaO-NP plants.

The immediate product of the PaO-catalyzed opening of the pheophorbide *a* ring, red chlorophyll catabolite (RCC), has phototoxic properties as well (Pružinská *et al.*, 2007). As RCC is the last phototoxic catabolite in the chlorophyll breakdown pathway, this compound is the likely cause of cell death in PaO-NP lines during natural senescence. Previous work has suggested a potential interaction between PaO and RCCR, the enzyme immediately downstream of PaO, which rapidly reduces RCC to pFCCs. (Pružinská *et al.*, 2007). These two enzymes function almost simultaneously in the chlorophyll degradation pathway, therefore the notion that they could a interact to form a protein complex is highly plausible. Should this be the case, a mutation in the PaO protein could alter the ability of PaO and RCCR to interact, perhaps in turn reducing RCCR functionality. Impaired RCCR activity would lead to RCC accumulation in leaf tissue, ultimately triggering a cell death reaction in the PaO-NP plants, similar in appearance to the one seen in the PaO-P mutant line.

CHAPTER 3 FIGURES AND TABLES

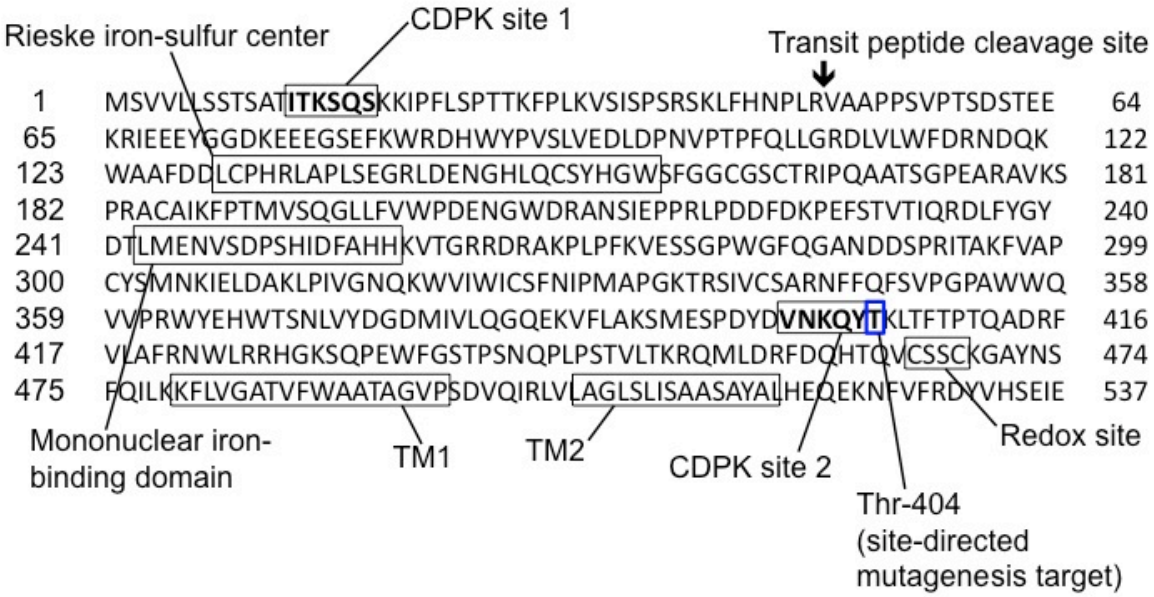

Figure 1. Codon-derived amino acid sequence of the *Arabidopsis thaliana* pheophorbide *a* oxygenase protein (AtPaO). Both putative CDPK target sequences (in bold) containing phosphorylatable T/S residues are highlighted. As indicated, Thr-404 (shown in blue) of the second CDPK site was selected for site-directed mutagenesis. Also noted are the chloroplast transit peptide cleavage site, Rieske iron-sulfur center, mononuclear iron-binding domain, and two transmembrane domains (TM1 and TM2). Additionally, a redox-active site near the carboxyl-terminal end of the protein is indicated. All sites highlighted here are conserved between Arabidopsis and both canola (BnPaO1 and BnPaO2) proteins.

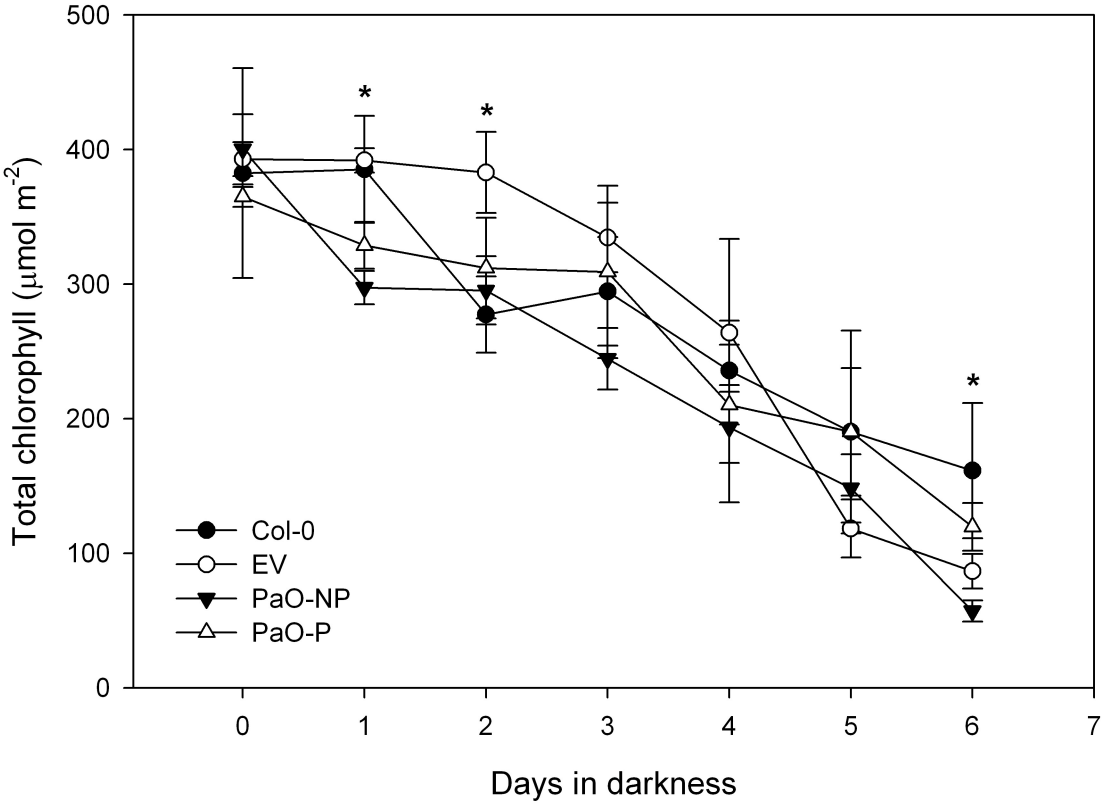

Figure 2. Chlorophyll content in senesencing leaves. Total chlorophyll levels were calculated on a per leaf area basis. Leaf punches were taken prior to, and throughout the course of artificial senescence, which was induced by incubating detached leaves in total darkness. Chlorophyll levels in pre-senescent leaf tissue were similar in all lines assayed, as seen at day 0. As senescence progressed, PaO-NP displayed a slightly accelerated rate of chlorophyll degradation, first seen at day 1. By day 7, chlorophyll content in all lines had decreased by at least 50%, though chlorophyll was statistically lower in PaO-NP than in all other lines. ($P < 0.05$ [*])

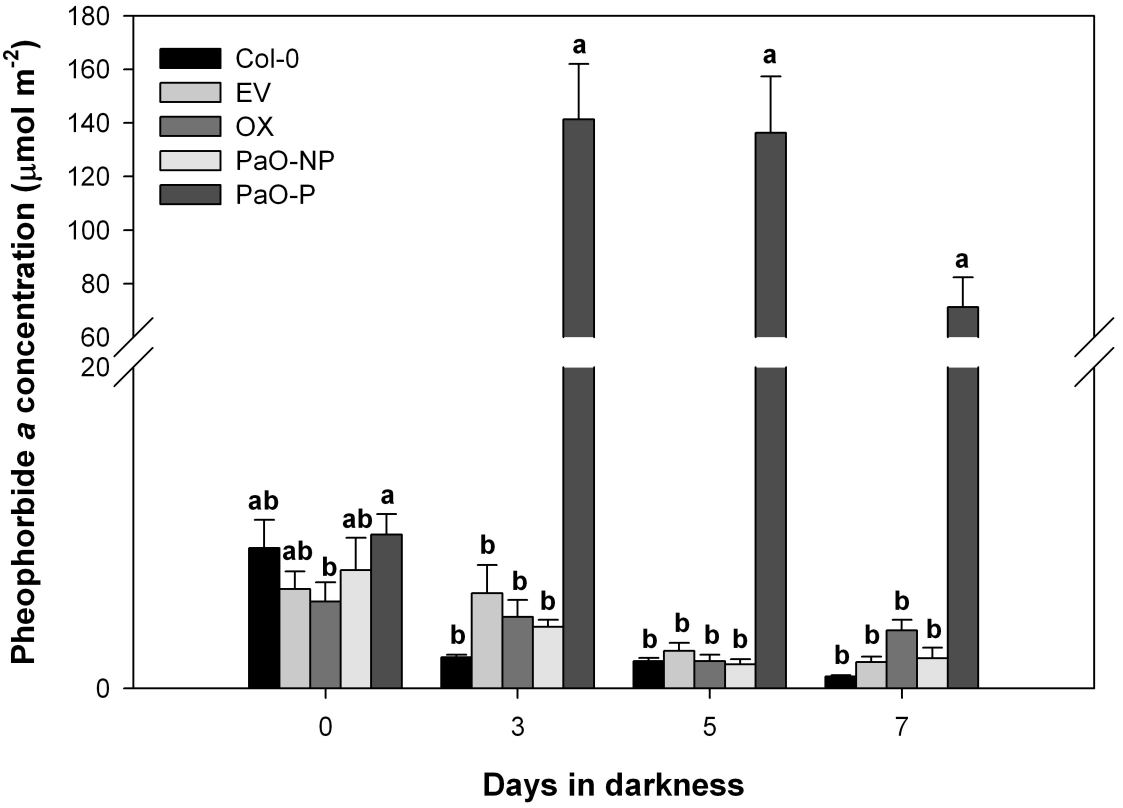

Figure 3. Accumulation of pheophorbide *a,* the phototoxic substrate of PaO, in senescing leaves. Green polar chlorophyll catabolites were extracted from detached leaves (n=6) at four timepoints before and during artificially-induced senescence, and pheophorbide *a* concentrations were determined by LC-MS/MS. Mutant and control lines had mostly similar pheophorbide *a* levels prior to senescence, although levels in the OX line were statistically lower than those in PaO-P at day 0 ($P < 0.05$ [a, b]). By day 3, pheophorbide *a* levels had decreased in all lines with the exception of PaO-P, in which pheophorbide *a* had accumulated to a concentration roughly 30-fold higher than that of the other lines ($P < 0.0001$ [a, b]). Pheophorbide *a* levels were significantly higher in PaO-P leaves throughout the remainder of the senescence regime ($P < 0.0001$ [a, b]).

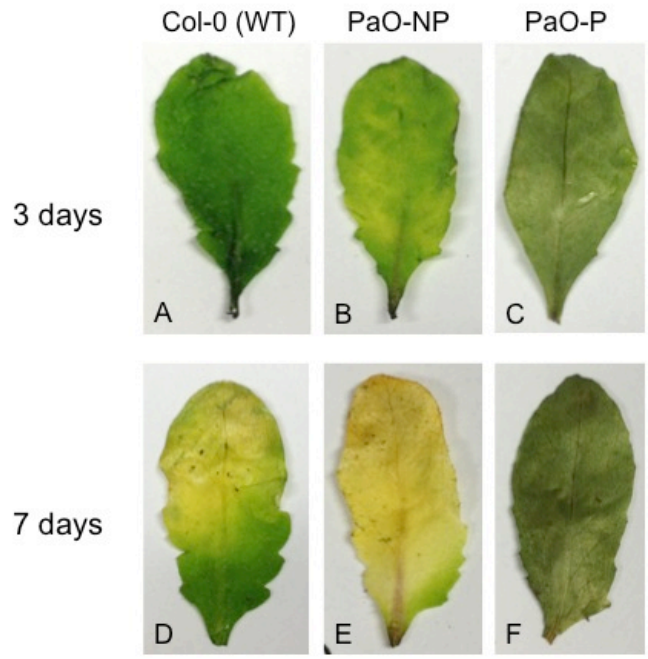

Figure 4. Degreening in senescing Arabidopsis leaves. Differences in chlorophyll content between lines could be visually detected. Attached leaves were incubated in darkness by covering with aluminum foil sleeves for periods of 3 and 7 days. WT and PaO-NP lines showed visible signs of yellowing after 3 days in darkness (A, B), and after 7 days few green areas remained (D, E). PaO-P leaves did not yellow, but rather were green and brittle in appearance (C, F), suggesting that chlorophyll or one of its green catabolites was accumulating during dark-induced senescence, likely the result of a lesion in the chlorophyll degradation pathway.

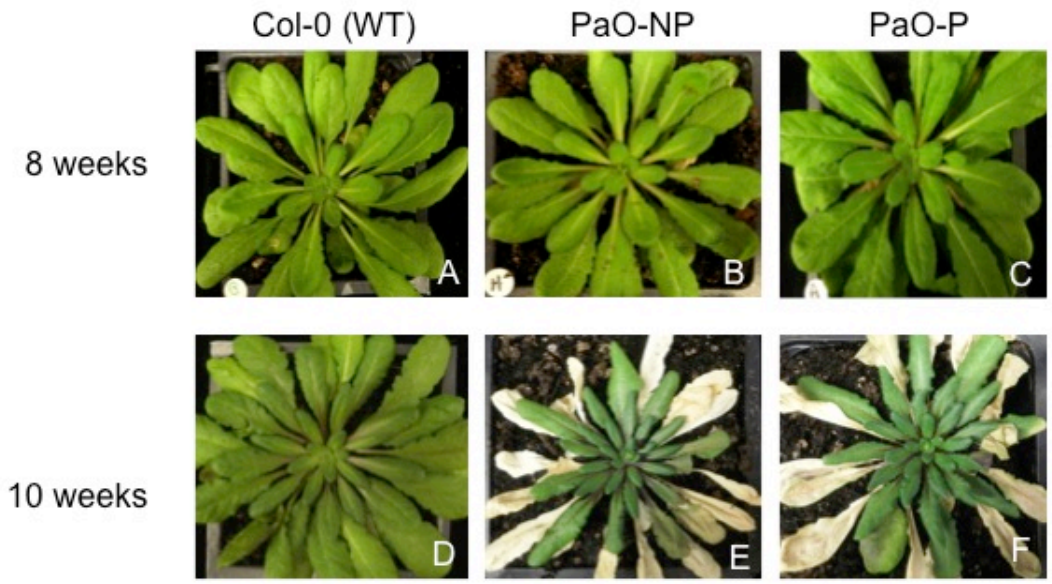

Figure 5. Whole plant appearance after 8 and 10 weeks of development under normal, short-day chamber growth conditions. Wild-type Col-0 plants had similar appearances at both 8 and 10 weeks (A, D). PaO-NP and PaO-P plants displayed a strong cell death phenotype after 10 weeks of growth (E, F), which appeared to be leaf age-dependent, as lesions formed first in older leaves. Cell death in these mutant lines was likely caused by the presence of phototoxic intermediates formed during chlorophyll degradation. In the case of PaO-P, pheophorbide *a* accumulated in senescing leaf tissue, as indicated by LC/MS-MS analysis. Cell death lesions in PaO-NP plants were likely due to the accumulation of RCC.

CHAPTER 4

DISCUSSION

Chlorophyll degradation is a vital component of the senescence stage of development in higher plants. Comprised of a rapid and highly systematized cascade of enzyme-mediated catabolic steps, the chlorophyll breakdown process functions to ensure the safe disposal of photoactive compounds before their cytotoxic effects can be expressed in senescing plant tissues. In recent years, an appreciable amount of effort has gone towards characterizing the steps involved in chlorophyll degradation, however, information regarding regulation of this pathway at the post-translational level is lacking. Within the chlorophyll catabolic framework, the opening of the chlorin macrocyle, catalyzed by pheophorbide *a* oxygenase, has long been considered a critical regulatory point, emphasizing the need for a better understanding of the mechanisms governing PaO activity. Here, we demonstrated that PaO activity is at least partially mediated by the reversible phosphorylation of a threonine residue situated within a putative CDPK site near the enzyme's C-terminal region.

By exploring how phospho-site substitutions on PaO affect several parameters in senescing Arabidopsis leaves, a role for phosphorylation in regulating enzyme activity was recognized. The most conclusive evidence indicating that phosphorylation regulates PaO activity was provided by the quantification of pheophorbide *a* concentrations in senescing leaf tissue. LC-MS/MS analysis of pheophorbide *a* in the mutant plant lines revealed that leaves from the PaO-P line, which mimics the phosphorylated state at Thr-404, accumulate and retain remarkably high levels of this compound during senescence (Fig. 3). By contrast, pheophorbide *a* was not retained at any appreciable level in leaves from the

mutant line mimicking the dephosphorylated state at Thr-404, but rather decreased throughout senescence, in a pattern identical to that seen in the wild-type Arabidopsis line (Fig 3). That pheophorbide *a* was detectable at such elevated levels in the PaO-P leaves implies that the ability of the PaO enzyme to catabolize this compound is compromised in this mutant line. Furthermore, because the breakdown of pheophorbide *a* was essentially unaffected in PaO-NP mutants, it is very likely that the impaired enzyme activity apparent in the PaO-P plants was elicted by the specified mutation mimicking phosphorylation at Thr-404 on this PaO protein. From the pheophorbide *a* data alone it can be concluded that phosphorylation at this particular phospho-site on PaO drastically reduces the enzyme's ability to catalyze the conversion of PaO to RCC.

This notion that PaO activity is strongly reduced by phosphorylation at Thr-404, as suggested by the elevated pheophorbide *a* levels in PaO-P mutant plants, is further supported by visual observations of leaf appearance during senescence. PaO-P leaves incubated in darkness retained their green color (Fig. 4, C and F) , while PaO-NP (Fig. 4, B and E) and wild-type (Fig. 4, A and D) leaves exhibited distinct yellowing. Naturally senescing PaO-P plants, grown under lights in chambers, formed clear cell death lesions on rosette leaves (Fig. 5F). The dark-induced stay-green phenotype and the light-induced cell death phenotype, both seen here in PaO-P leaves, are characteristic of well-known functional knockdown lines (*e.g.,* Arabidopsis mutant *acd1*) whose gene encoding PaO is mutated (Pružinská et al., 2003). It follows that if the PaO-P line is behaving in a manner similar to that typical of a PaO knockdown mutant line, this enzyme is almost certainly functionally impaired by the phospho-site substitution.

Although the results of this study provide convincing evidence that PaO activity is influenced by the phosphorylation state of Thr-404, and that phosphorylation at this residue has substantially detrimental effects on enzyme activity, it is unclear exactly how PaO is affected by dephosphorylation at this site. As the capacity of the PaO enzyme to catabolize its substrate is greatly reduced by phosphorylation, dephosphorylation would be expected to have the opposite effect, leading to enhanced enzyme activity; however, such an effect was not readily recognizable here. Measurements of total chlorophyll content overtime in senescing leaves suggested that chlorophylls *a* and *b* were being degraded at a slightly accelerated rate in the nonphosphorylation mimic mutant plants, yet concentrations of leaf chlorophyll in this line were not consistently significantly lower than in other lines (Fig. 1). Moreover, because chlorophyll is not directly catabolized by PaO, leaf chlorophyll content is not an especially reliable method of estimating PaO activity.

Visual observation of yellowing on attached leaves that were covered with foil sleeves to induce senescence was another simplified means by which PaO activity was approximated in the mutant lines. Leaf yellowing was seemingly more pronounced and rapid in the PaO-NP mutant leaves (Fig. 4, B and E) than in wild-type (Fig. 4, A and D), especially midway through senescence, suggesting a somewhat enhanced rate of chlorophyll catabolism in plants mimicking the non-phosphorylated state of PaO. The implication of elevated chlorophyll catabolic capacity in Pa0-NP plants based on leaf yellowing, however, was not verified by pheophorbide *a* quantification. Pheophorbide *a* concentrations in PaO-NP leaf tissue, which reliably reflect PaO activity, did not decrease more rapidly or to a greater extent than in the wild-type control line or the PaO over-expresser (OX) control line (Fig. 3). Hence, while an explicit decrease in the functional

ability of PaO – most likely due to phosphorylation of Thr-404 – was easily identified here, we were unable to demonstrate through this work alone whether PaO activity was enhanced by dephosphorylation at the given site.

If reversible phosphorylation at Thr-404 were the only mechanism regulating PaO, and phosphorylation at this site reduced enzyme activity, then dephosphorylation would predictably increase activity, which was not the apparent case. Thus, it is more likely that several post-translational mechanisms contribute to the control of pheophorbide *a* oxygenase, and that an interaction effect might exist between these mechanisms. One such process that may regulate PaO activity, in conjunction with phosphorylation, is a thioredoxin (Trx)-mediated redox reaction. It has been shown *in vitro* that a redox-active motif (CxxC) near the C-terminal region of the PaO protein interacts with Trxs *f* and *m*, which in turn influences activity considerably (Bartsch *et al.*, 2008). Although this study was performed *in vitro*, it seems highly plausible that the redox-active site would also play a role in regulating PaO activity *in planta*. Should this be true, the predicted heightened ability of the PaO-NP mutant line to catabolize pheophorbide *a* would not have been observed for a few reasons. If the redox site does, in fact, contribute to enzyme activity, the PaO mutant mimicking the nonphosphorylated state at Thr-404 might not be fully activated unless the redox site is also in an active state. Alternatively, the potential exists for an interaction to occur between the Trx and CDPK target sites. Changes in the phosphorylation state of Thr-404, by altering protein configuration, could affect how these sites interact and, subsequently, the overall ability of PaO to function. As either of these scenarios is feasible, future studies exploring the effects of combinations of mutations at the redox-active and CDPK phosphorylatable sites on PaO activity might prove worthwhile.

Also worth considering when interpreting the effects of phosphorylation on PaO activity during senescence, is whether the phosphorylation status of Thr-404 affects the ability of PaO to interact with other enzymes in the chlorophyll degradation pathway. For example, previous work has suggested that PaO and RCCR, the enzyme directly downstream of PaO, interact very closely, possibly forming a protein complex during chlorophyll degradation (Rodoni et al., 1997; Pružinská et al., 2007). Conformational changes on the PaO protein, mediated by the presence or absence of a phosphate group (or a mutation mimicking the presence or absence of a phosphate group) on Thr-404 might profoundly alter the capacity for such a complex to assemble. The observation in this study that PaO function is not necessarily enhanced by dephosphorylation, despite being impaired by phosphorylation, could perhaps be explained by a reduced ability of nonphosphorylated PaO to interact with RCCR. As formation of a complex between these two enzymes is thought to maximize their individual functional capacities through metabolic channeling (Hörtensteiner, 1999), a modification hindering this interaction between PaO and RCCR would predictably decrease enzyme activity. Accordingly, if the PaO enzyme were in a hypophosphorylated state, as is the case in the PaO-NP mutant line, it might not be capable of effectively forming a protein complex with RCCR and, as a result, an elevated level of enzyme activity would not be seen.

Additional evidence supporting this theory, that the ability of PaO to interact with RCCR may be restricted in the nonphosphorylated mutant line, can be found in the phenotypic observations of naturally senescing PaO-NP plants in this study. Whereas the cell death lesions seen in PaO-P plants (Fig. 5F) were undoubtedly a result of pheophorbide *a* accumulation in senescing leaf tissue, the source of this unexpected phenotype in PaO-NP

plants (Fig. 5E) was unclear. Since pheophorbide *a* was shown to be adequately catabolized in the PaO-NP line, accumulation of the next – and last – phototoxic catabolite in the chlorophyll breakdown pathway, RCC, is most likely the cause of cell death in these plants. If PaO were unable to interact with RCCR, activity level of RCCR would potentially be impaired – as shown by Pružinská et al. (2007) – and result in the accumulation of RCC in senescing tissue. In the presence of light this compound would elicit cell death in PaO-NP leaves. Analyses to quantify RCC levels in senescing leaf tissue will eventually need to be performed in order to verify whether RCCR activity is indeed influenced by the phosphorylation state of PaO.

The idea that the ability of PaO to interact with other proteins during chlorophyll degradation might depend on its phosphorylation state, can be taken a step further and considered in the context of a novel chlorophyll degradation model, recently proposed by Sakuraba et al. (2012). Their work suggests that all of the chlorophyll catabolic enzymes are recruited by SGR to LHCII in the thylakoid membrane, where they form a complex and interact to metabolically channel chlorophyll catabolites through the degradation pathway, as a means of preventing phototoxicity in the senescing plant. Assuming phosphorylation does influence how PaO interacts with other proteins, and should this proposed model prove to be correct, the network of factors contributing to PaO activity level during senescence would become vastly more complex. The effects of phosphorylation would likely vary depending on the enzyme with which PaO is interacting, and the ability of PaO to function in this enzyme complex might be altered by the presence or absence of phosphate groups on amino acid side chains. Additionally, the phosphorylation state of PaO could in turn affect activity levels of other chlorophyll catabolic enzymes, ultimately

having an influence on the metabolic channeling capabilites of the enzyme complex altogether.

Regardless of the many potential mechanisms by which phosphorylation status might affect PaO activity and its capacity to interact with other proteins, the data presented here indicate that phosphorylation at a defined CDPK site drastically reduces functional ability of the enzyme, providing clear evidence of a role for phosphorylation as a principal mechanism regulating pheophorbide *a* oxygenase. Consequently, this information can be used to eventually acquire a better understanding of the various scenarios under which chlorophyll catabolism is impaired during senescence.

One such scenario is the green seed problem in canola, characterized by an interruption in seed chlorophyll catabolism following frost exposure during the growing season. The retention of green color in mature canola seeds is thought to be the result of a cold-induced impairment in PaO activity (Chung et al., 2006). Despite speculation that a CDPK-mediated phosphorylation mechanism could be contributing to the reduction in enzyme activity, it was not previously known whether phosphorylation at candidate CDPK target sites on this enzyme plays an actual role in regulating PaO. Here, we were able to demonstrate that phosphorylation at a specified CDPK recognition site does indeed strongly hinder the enzyme's functional capabilities.

Since cold-stress triggers a transient increase in Ca^{2+} levels in plant cells (Gilroy and Trewevas, 1994), and Ca^{2+} activates CDPKs, then the Ser/Thr residues at CDPK target sites would predictably become hyperphosphorylated when plants are exposed to freezing temperatures, as was demonstrated (Chung et al., 2006). Our data, indicating that a mutation mimicking the phosphorylated state at a candidate CDPK recognition site greatly

reduces the ability of PaO to catabolize pheophorbide *a*, support prior speculation that PaO activity may be attenuated by phosphorylation. Thusly, the results of this study strengthen the case for a specified series of events thought to lie at the foundation of the canola green seed problem; that is, a cold-induced increase in calcium levels – as would be triggered by an early season frost – activates CDPKs, which phosphorylate the PaO protein, in turn reducing PaO activity and chlorophyll catabolism altogether, resulting in green seed at harvest. Although this scheme of events is highly plausible, a stronger understanding of the cause of the green seed problem will ultimately be necessary in order to identify potential solutions to this largely detrimental phenomenon that is commonplace in the commercial canola industry.

CONCLUSIONS

Taken together, the results of this work strongly imply a role for phosphorylation in regulating the chlorophyll catabolic system of higher plants during senescence. The *in vivo* functional capacity of PaO, which catalyzes the key regulatory step in the chlorophyll degradation pathway, is altered considerably by the introduction of amino acid substitutions at a predicted phosphorylatable site on the enzyme. That PaO is unable to effectively catabolize its substrate, pheophorbide *a*, in senescing leaves of mutant Arabidopsis lines mimicking a phosphorylated state, provides compelling evidence to signify that PaO activity is impaired by phosphorylation at this defined site. While potential mechanisms by which the presence of a phosphate group might hinder PaO activity can be speculated upon, further studies will be necessary to develop a model describing how this post-translational modification regulates PaO and the chlorophyll catabolic pathway as a whole.

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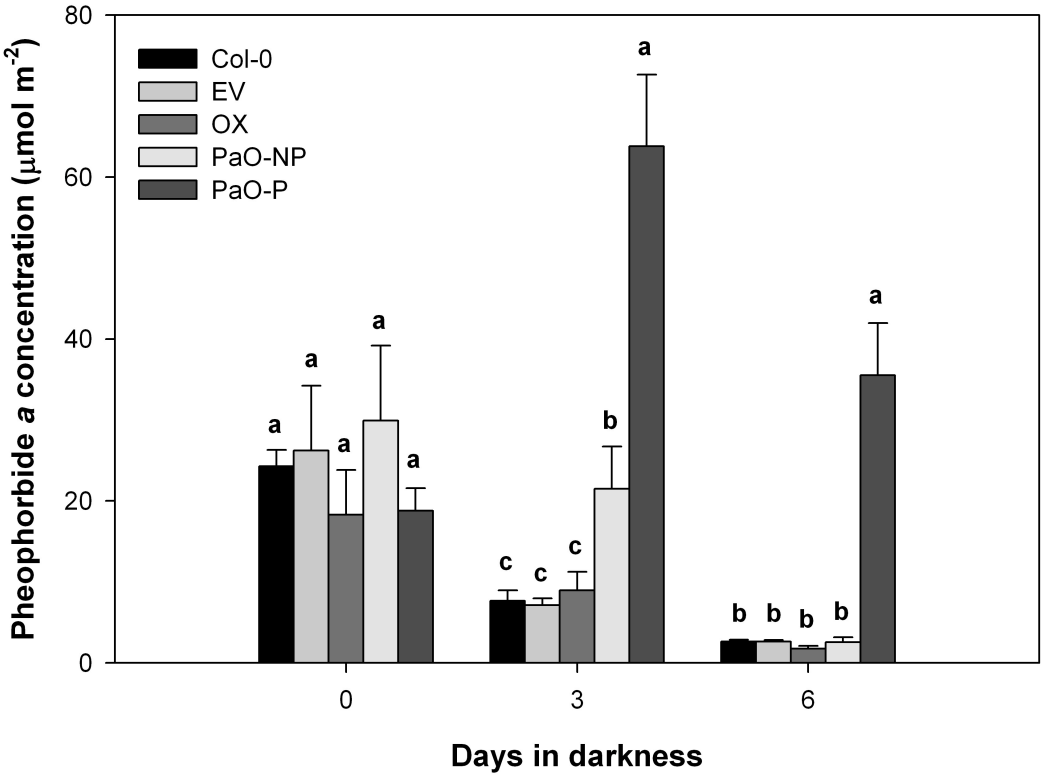
APPENDIX



Supplemental Figure 1. Accumulation of pheophorbide *a* during senescence. Experiments described in Figure 3 were repeated, yielding analogous results. Pheophorbide *a* levels were similar in all lines at 0 days in darkness. Concentrations decreased steadily throughout senescence in the PaO-NP and control lines, whereas in PaO-P, leaves had accumulated pheophorbide *a* midway through senescence, to levels 3-fold higher than those observed at day 0. Pheophorbide *a* in the PaO-P line had decreased by the end of senescence, though final concentrations were still roughly 7-fold higher than those in all other lines. This trial confirms what was previously observed, providing further evidence that phosphorylation of PaO at Thr-404 greatly reduces functionality of the enzyme.